Evaluation of Gossypetin and Avicularin in Alzheimer's disease and associated conditions

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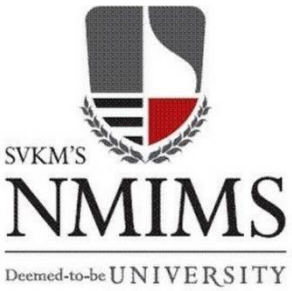

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BACKGROUND OF THE STUDY

Alzheimer's disease (AD) is an age-dependent neurodegenerative disorder and the leading cause of dementia characterized by the progressive loss of neurons, which typically leads to severe cognition impairment and memory dysfunction in the worldwide population older than 65 years of age (50–70% of all dementia cases) [1]. According to, Alzheimer's association 2019 report approximately 5.8 million Americans are living with Alzheimer's dementia in the United States. In absolute terms, AD affects up to 35.6 million people worldwide and 7.7 million new cases of dementia added every year [2]. The pathophysiology of AD is related to neuronal death, which initiates in the cortex and hippocampus brain region. Currently, AD is the sixth-leading cause of death in the United States as well as a major cause of morbidity and economic burden worldwide [3,4]. It is predicted that 70 to 80 million people would suffer from Alzheimer's disease-related dementia by 2030, with the overall cost of caring for these people reaching almost $2 trillion. In the paucity of efficient and promising therapies, the projected number of people worldwide with dementia may reach 115 to 131.5 million by 2050 [5,6].

The pathogenesis of AD has been characterized by the accumulation of Aβ oligomers, Aβ plaques in the extracellular region of brain cells, an alteration in synaptic function, loss of synapse, change in the function of neurotransmitters, alteration in proinflammatory or inflammatory responses, and neuronal death [7,8]. An additional hallmark of AD is hyperphosphorylated tau protein (microtubule-associated protein) which is found mainly in the cortical and hippocampal regions of the brain [9,10]. In the CNS, tau protein plays a vital role in stabilizing microtubules and promoting tubulin assembly into the microtubule. Moreover, the deposition of excessive Aβ oligomers and plaques has been reported to trigger excessive hyperphosphorylation of tau, which forms intracellular neurofibrillary tangles (NFTs) in AD pathology [11]. The intracellular neurofibrillary tangles lead to dendritic spine collapse and cause microtubule disassembly, which is further responsible for the degeneration of neurons

[12]. Intracellular NFT, extracellular Aβ plaques, Aβ oligomers, and soluble tau forms are the initial factors responsible for cognitive impairment. The main characteristics of AD patients include memory loss or impaired memory and thinking, disorientation and confusion, misplacing things, trouble in performing familiar tasks, changes in personality and behaviour, inability to follow directions, impaired visual and spatial skills, loss of normal sleep patterns, difficulty in doing daily tasks and poor judgment [13]. Moreover, patients with dementia lose the ability to independently perform daily activities [14]. Additionally, oxidative stress, mitochondrial dysfunction, excessive reactive oxygen species production, lipid peroxidation, proteasomal dysfunction, microglial activation, neurotransmitter alteration, and neuroinflammation have been established as major conditions associated with AD [15]. However, its cause remains poorly understood, and there is no cure for AD despite extensive research and billions of dollars spent over decades.

Currently marketed drugs for AD includes Acetylcholinesterase inhibitors (AChEI) and N-methyl-D-aspartate receptor (NMDAR) antagonists and a combination of memantine and donepezil have been approved by the US Food and Drug Administration (FDA) for the management of AD. Rivastigmine, donepezil, and galantamine belong to the AChEI group, and memantine is an NMDAR antagonist [16, 17].

Several mechanisms and downstream pathway involved in the pathogenesis of Alzheimer’s Disease, from which MAPK is one of them. Mitogen-activated protein kinase (MAPK) p38 has been implicated in the pathogenesis of Alzheimer's disease, but the upstream cascade leading to p38 activation has not been elucidated in the disease [18]. In the present study, we focused on mitogen-activated protein kinase kinase 6 (MKK6), one of the upstream activators of p38 MAPK. We found that MKK6 was not only increased but also specifically associated with granular structures in the susceptible neurons in the hippocampus and cortex of Alzheimer's disease patients but was only weakly diffuse in the cytoplasm in neurons in control cases [19].

Immunoblot analysis demonstrated a significant increase of MKK6 level in Alzheimer's disease compared with age-matched controls. In this regard, in hippocampal and cortical regions of individuals with Alzheimer's disease, the activated phospho-MKK6 was localized exclusively in association with pathological alterations including neurofibrillary tangles, senile plaques, neuropil threads and granular structures, overlapping with activated p38 MAPK suggesting both a functional and mechanic link [20].

Flavonoids are found in various dietary sources and exhibit several biological activities, including antioxidant, anti-inflammatory, anti-viral, and anti-cancer effects. They have been considered as good candidates for cancer prevention. Gossypetin (3,5,7,8,3',4'hexahydroxy flavone) is found in the flowers and the calyx of Hibiscus sabdariffa (roselle). Gossypetin has been reported to exert anti-mutagenic, anti-atherosclerotic, and antioxidant activities. Here we provide evidence showing that gossypetin is a potent MKK3 and MKK6 inhibitor and apart from this gossypetin may show neuroprotective action via targeting MKK-6 [21].

Furthermore, Avicularin is an active phytoconstituent with several health benefits. Avicularin (quercetin-3-alpha-l-arabinofuranoside) is a bioactive flavanol, isolated from several plants including Polygonum aviculare, Rhododendron aureum, and Taxillus kaempferi [22]. The previous literature search revealed that avicularin possesses anti-oxidant, anti-inflammatory, and anti-depressant activity [23]. According to the study by Vo et al., avicularin reversed the inflammation induced by lipopolysaccharides by inhibiting the production of nitric oxide, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) [24]. Avicularin has also been reported to ameliorate human hepatocellular carcinoma, rheumatoid arthritis via MEK/NF-κB pathway activation [25]. Furthermore, avicularin also inhibited the accumulation of intracellular lipids through repression of glucose transporter-4 mediated glucose uptake [26]. Interestingly, avicularin also exhibited antidepressant-like effects on CUMS-induced depression.

The ERK pathway can be activated by two main receptors, i.e., receptor tyrosine kinase (RTK) (i) and G-protein coupled receptor (GPCR)(ii). In addition, cytosolic mechanisms can also activate the pathway through protein kinase C (PKC) (iii) or calcium-calmodulin (CaM) (iv). The activation of the pathway can be directed by scaffolding proteins that hold together the components of the pathway. Once ERK is phosphorylated, cytoplasmic or nuclear changes may be induced, activating sets of genes responsible for neural differentiation, migration, the final fate, and plastic changes. Cytoplasmic p-ERK can promote processes such as apoptosis and neurogenesis [27]. By considering beneficial actions of avicularin through activating ERK, we have selected avicularin for our research study.

2. RATIONALES

Alzheimer's disease (AD) is the most common type of dementia. It causes severe suffering for the patients, in the form of progressive behavioral and neurological changes that include functional impairment, loss of independency, emotional problems and behavioral disturbances. In our research study, we have targeted three hallmarks of Alzheimer's Disease i.e. Amyloid beta, GSK-3β and cognition impairment. To induced amyloid beta, we have used $A\beta_{(1-42)}$ oligomer, further to increased levels of GSK-3β in the brain we have used and trimethyltin chloride (neurotoxin chemical) and chronic stress to induced cognition impairment and then by utilising previous potential activities of avicularin and gossypetin we have evaluated neuroprotective activity of both the compounds against Alzheimer's Disease [28].

2.1. Gossypetin

The Hibiscus species provides the very economical source of Gossypetin (GTIN) and related compounds. It was originally isolated from Hibiscus vitifolius (tropical rose mallow; also called Japa or Karupatti) and H. furcatus (Panchavam in Malayalam). The extracts of these plants are traditionally used for the treatment of diabetes, jaundice, inflammation [29]. These plants had

been shown to have potent antioxidant activity. According to prior research, gossypetin has important pharmacological activities such as anti-oxidant and anti-depressant, therefore it was hypothesised that gossypetin may reverse the deleterious effects produced by various chronic stressors and might promote neurogenesis by inhibiting QA production, accelerating BH4 synthesis and could be useful in the treatment of stress associated dementia. Further, research studies also demonstrated that the $A\beta_{(1-42)}$ accumulation initiates a series of cellular cascades including glial cell (microglia and astrocytes) activation [30]. The exact mechanism behind Aβ induced neuroinflammation hypothesis is still unknown. However, oxidative damage and mitochondrial dysfunction are responsible for shifting the cell signaling pathways toward proinflammatory and pro-apoptotic signals creating a link between oxidative stress and neuroinflammation [31]. Further, it was demonstrated that gossypetin is a potent MKK-6 inhibitor and also possess anti-inflammatory activity against proinflammatory cytokines. By considering, current evidence and essential actions of gossypetin, the present study demonstrated activity of gossypetin against trimethyltin chloride induced neuroinflammation, Amyloid beta induced Alzheimer's Disease and CUS induced cognition impairment [32].

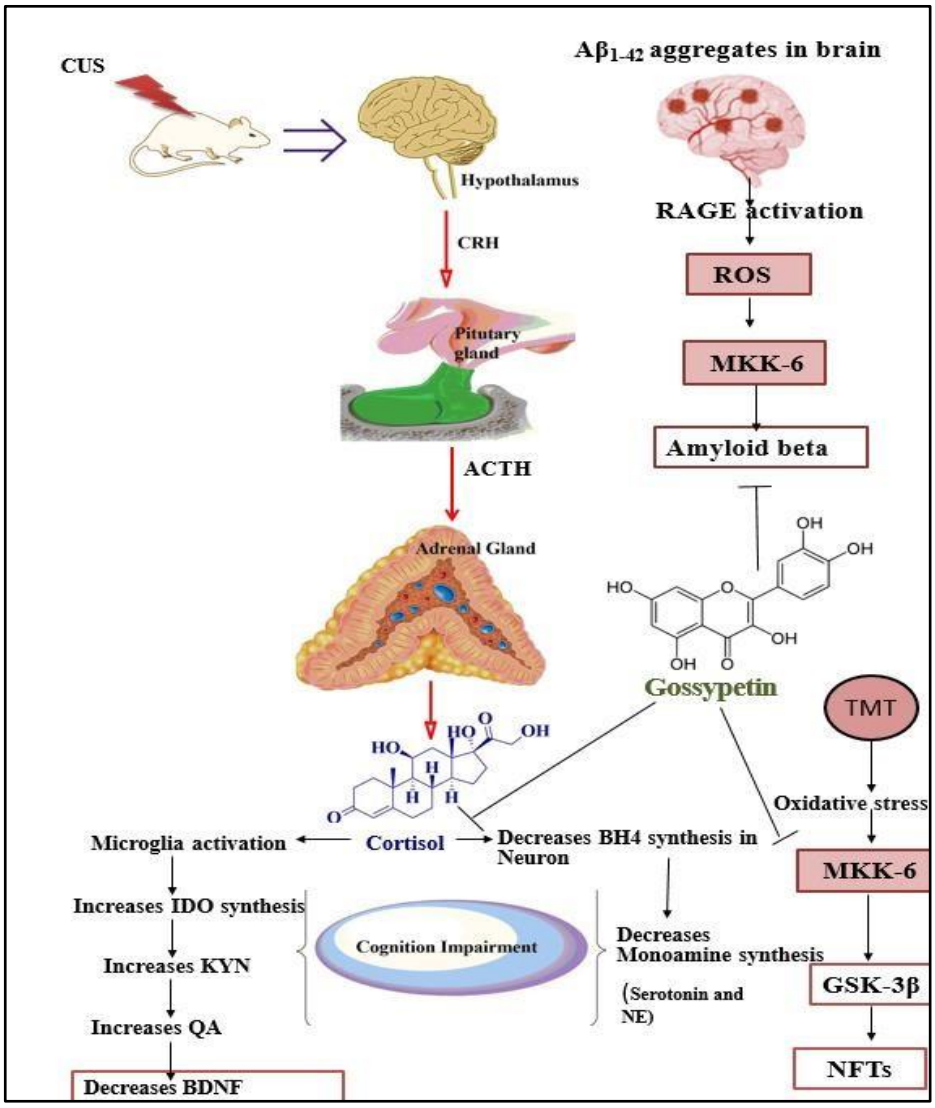

Figure 1: Mechanism of action of Gossypetin

2.2. Avicularin

Avicularin is an active phytoconstituent with several health benefits. Avicularin (quercetin-3alpha-l-arabinofuranoside) is a bioactive flavanol, isolated from several plants including Polygonum aviculare, Rhododendron aureum, and Taxillus kaempferi. The previous literature search revealed that avicularin possesses anti-oxidant, anti-inflammatory, and anti-depressant activity. According to the study by Vo et al., avicularin reversed the inflammation induced by lipopolysaccharides by inhibiting the production of nitric oxide, inducible nitric oxide synthase

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Further, to evaluate activity of avicularin against amyloid beta induced neurotoxicity. In vitro and in vivo experimental paradigms have demonstrated the neurodegenerative activity of the Aβ peptide. Primarily, amyloid-beta plaques also cause excessive oxidative stress, increased acetylcholinesterase activity, and decreased levels of brain-derived neurotrophic factors [35]. Excessive aggregation of Aβ causes deleterious events like endoplasmic reticulum stress, aberrant tau phosphorylation, disrupt Ca^{2+} homeostasis, and synaptic dysfunction. Previous literature search has shown that intracerebroventricular injection of Aβ causes brain dysfunction and plays an integral part in the progression of Alzheimer's disease by activating RAGE (receptor for advanced glycation endproducts) [36]. RAGE is the multiligand receptor present on neuronal and non-neuronal cells and plays an essential role in an inflammatory response. Activated RAGE further complicates the disease condition in two ways 1) increasing inflammatory cytokines via NF-κB pathway stimulation and 2) excessive production of reactive oxygen species [37].

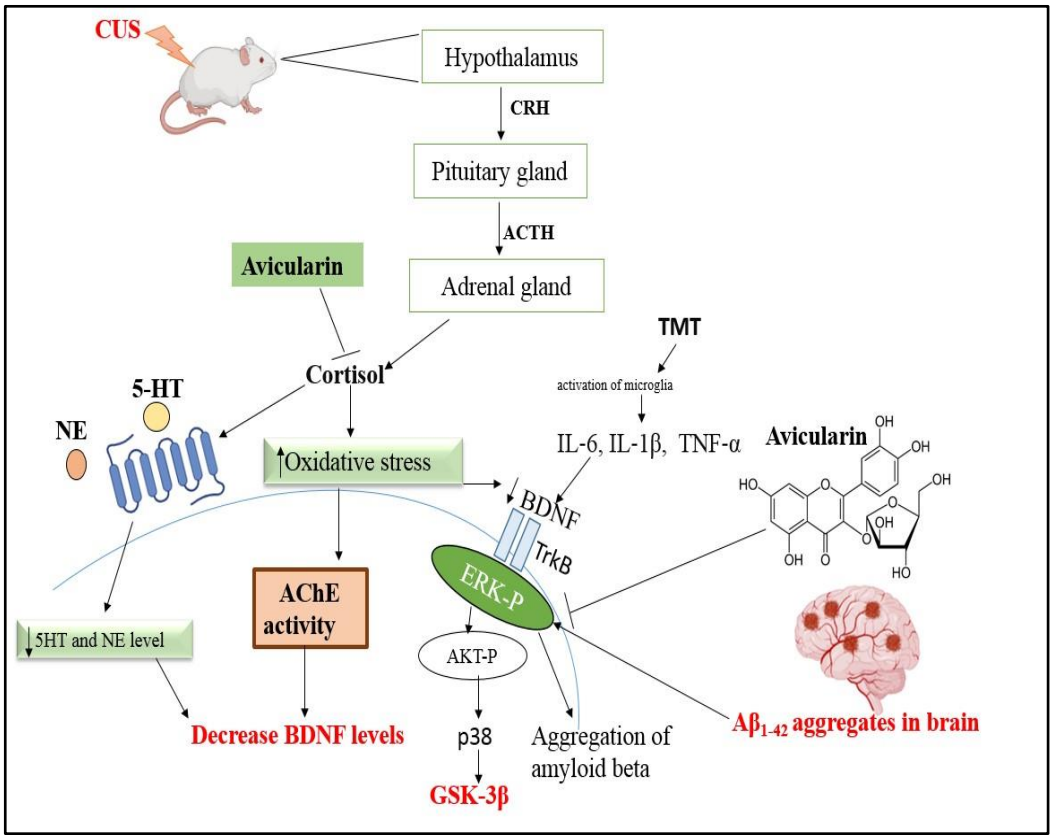

Figure 2: Mechanism of action of Avicularin

This unnecessary activated signaling pathway enhanced neuroinflammation and oxidative stress and contributed to neurodegeneration. Amyloid-beta was shown to be the significant element; however, other amyloid beta-induced factors can play an essential role in exacerbating AD and causing additional comorbidities. Therefore, preventing Aβ induced neurotoxicity is essential to improve cognition [38]. Hence, by considering overall actions of avicularin on ERK, we have measured effect of avicularin against amyloid beta induced Alzheimer's Disease [39].

Further, TMT-induced neuroinflammation and its consequences have been shown to be a helpful tool for research [40]. Furthermore, TMT can increase glycogen synthase kinase (GSK-3β) levels through activating the serine/threonine protein kinase/ mitogen activated protein kinase (ERK/MAPK) pathway, according to research by Kim et al. TMT activates microglia, which stimulates the pro-inflammatory cytokines [41]. These activated microglia are

responsible for production of inflammatory mediators (IL-6, IL-1β, and TNF-α). High levels of cytokines cause a low levels of BDNF in the brain, which reduces its binding to the tropomyosin receptor kinase B (TrkB) receptor. This stimulates ERK phosphorylation even further, resulting in higher GSK-3β levels. Furthermore, because of TMT's possible harmful effects on the brain, we chose a TMT-induced model for our research investigation [42]. By considering anti-inflammatory activity of avicularin and to identify the effect of avicularin against GSK-3β levels by targeting ERK, we have used trimethyltin chloride as a inducing agent.

3. OBJECTIVES

The objectives of present research work were:

- To study the effect of Gossypetin and Avicularin in Alzheimer's disease.
- To evaluate effect of Gossypetin and Avicularin on neuroinflammation in Alzheimer's disease.
- To evaluate effect of Gossypetin and Avicularin on chronic unpredictable stress induced cognition impairment**.**

4. APPROACHES TO THE ISSUES

There is currently no cure or disease-modifying treatment for Alzheimer's disease; nevertheless, FDA approved medicines provide solely symptomatic alleviation in individuals with the condition. NMDAR antagonist and cholinesterase inhibitors do not block the formation of Aβ plaques and NFTs, hence, they do not seize the progress of dementia. However, they lead to a temporary slowdown in the loss of cognitive function [43]. Interestingly, many drug candidates reached various clinical trial phases, but due to adverse effects and lack of therapeutic efficacy, most of the compounds did not succeed in Phase II/III trials. Therefore, understanding the comprehensive pathogenesis of AD is required before focusing on novel drug development. Moreover, the attention in AD drug development is also shifting from treatment to prevention [44]. However, new strategies seem to focus on decreasing the production, aggregation, misfolding of Aβ proteins, tau, and increase in clearance of toxic aggregate or misfolded forms of these proteins based on the previous clinical and nonclinical studies. Targeting oxidative stress, neuroinflammation, neurotransmitter signalling, and genetic factors may also help indirectly to influence Aβ or tau accumulation [45].

Synthetic drugs have limitations like side effects, treatment cost, prolonged and daily medication which develop an urge to find out efficient economic and safe treatment against Alzheimer's Disease [46]. Plants have always played a crucial role from ancient times in the treatment of many life-threatening disorders. Natural products rich in alkaloids, glycosides, tannins, and flavonoids are gaining a lot of interest of researchers in the present time. Avicularin is a bioactive flavonoid, has antioxidant, anti-depressant, and anti-inflammatory properties [47]. Avicularin reduces inflammatory response by decreasing levels of proinflammatory cytokines (TNFα, IL-6, IL-1β) and suppresses the excessive synthesis of reactive oxygen species, nitric oxide, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) [48]. According to previous literature search, avicularin suppresses ERK phosphorylation while lowering p38

levels, which are ultimately responsible for cell proliferation, differentiation, and neurogenesis. By considering all vital action of avicularin, in our current research study, we have depicted neuroprotective action of avicularin [49].

Furthermore, Gossypetin is a flavonoid mainly isolated from hibiscus species. Gossypetin possess broad therapeutic activities like anti-oxidant, antidepressant and anti-atherosclerotic. According to *in-vivo* studies, gossypetin exhibits anxiolytic and antidepressant effects at different doses [50]. Furthermore, mice treated with gossypetin demonstrated anti-oxidant action. In-vitro investigations have also shown that gossypetin has anti-atherosclerotic properties by blocking LDL oxidation. According to prior research, gossypetin has important pharmacological activities such as anti-oxidant and anti-depressant, therefore it was hypothesised that gossypetin may reverse the deleterious effects produced by various chronic stressors, amyloid beta and GSK-3β and might promote neurogenesis and could be useful in the treatment of Alzheimer's Disease [51].

5. MATERIALS AND METHODS

5.1. Materials

Avicularin and Gossypetin procured from TCI chemicals, India. Biological kits were procured from Transasia Biomedicals Ltd. India. TNF-α, IL-6, IL-1β, corticosterone were procured from Krishgen Biosystems, India. BDNF, GSK-3β and amyloid beta procured from ABclonal Technology, USA and Santa Cruz Biotechnology, Inc, USA. Chemicals required for assessment of oxidative stress are procured from SD Fine chemicals, India and Sigma Aldrich, USA

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5.2. Methods

5.2.1. Acute oral toxicity study of Avicularin and Gossypetin

Acute oral toxicity study of avicularin and gossypetin was performed as per OECD guideline 423 with slight modifications (OECD, 2001). Female Sprague Dawley rats were divided into six groups containing three animals in each and received single-dose treatment.

Table 1: Grouping for acute oral toxicity study.

Group	Treatment
I	0.5% carboxymethyl cellulose (CMC) as a vehicle
II	Avicularin (300 mg/kg)
III	Avicularin (2000 mg/kg)
IV	Avicularin (5000 mg/kg)
V	Gossypetin (300 mg/kg)
VI	Gossypetin (2000 mg/kg)

Animals were observed individually after dosing for any sign of changes in behavior and physiological appearance during the first 30 min and then periodically during the first 24 h with special attention on the first 4 h. All the groups were kept under observation for 14 days. Various observations related to the physical appearance like change in skin and fur, eyes, lacrimal secretion were noted. Autonomic, somatomotor, behavioral patterns and respiratory abnormalities were also observed. Body weight, food and water intake were also determined till 14^{th} day. Animals were also observed for tremors, convulsions, salivation, diarrhoea, lethargy, sleep, and coma [52].

5.2.2. Repeated dose oral toxicity study of gossypetin and avicularin

28-days repeated dose oral toxicity study was performed as per OECD guideline 407. Sprague Dawley rats were divided into seven groups of 10 animals in each (5 Male and 5 female) and treatment was given for 28 days as showed in Table 7 [53].

Table 2: Grouping for repeated dose oral toxicity study

Group	Treatment
I	0.5% CMC as a vehicle
II	Avicularin (25 mg/kg)
III	Avicularin (50 mg/kg)
IV	Avicularin (100 mg/kg)
V	Gossypetin (10 mg/kg)
VI	Gossypetin (20 mg/kg)
VII	Gossypetin (40 mg/kg)

All animals were supplied with a standard pellet diet and water ad libitum throughout the study. Animals were observed frequently for a sign of toxicity and mortality. Blood was withdrawn from retro-orbital plexus and collected in EDTA (disodium ethylenediaminetetraacetic acid) containing tubes and subjected to haematological analysis. Then, plasma was separated by centrifugation for the determination of biochemical and electrolyte analysis. Gross necropsy and histopathological study were performed at the end of the study.

5.2.3.Pharmacokinetic study of avicularin and gossypetin

Avicularin absorption and BBB penetration rate were assessed using a pharmacokinetic study. Initially, method was developed in LC-MS/MS instrument. Acetonitrile:methanol (90:10) was taken as mobile phase, flow rate was maintained at 0.40 mL/min.

Carbamazepine was selected as internal standard, sample injection volume 10µl and C18 column (2.1 mm × 50 mm, 1.7µm particle size) was used for entire study (Buqui et al. 2015). After developing bioanalytical method in LC-MS/MS, total 42 male *Sprague Dowley* rats (three animals for each time point) were used. The protocol was approved by the Institutional Animal Ethics Committee (approval number- CPCSEA/IAEC/P-32(R)/2021). Food was prohibited for 12h before the experiment but water was freely available. Blood samples (0.3 mL) were collected from the retro-orbital plexus into heparinized 1.5ml polythene tubes at 0, 30, 60, 120, 240, 360, 480 and 24 hr after administration of avicularin. The plasma samples were extracted from collected blood samples and stored at -20ºC. Further, the plasma samples were processed and analyzed in the LC-MS/MS [54].

After measuring concentration of drug in plasma, we have also estimated the drug concentration in brain homogenate. Avicularin was given to the animals and at respective time point animals were sacrificed and brain tissue was collected at 0min, 60min, 120 min, 240min, 480min, 24hr and 48 hr. Isolated brain tissues were homogenized in phosphate buffer saline solution, collected brain supernatant was processed and analysed in LC-MS/MS. All the values are presented as mean ± SEM. All the data analysed by using Pksolver software [55].

Similar procedure was followed for the measurement of concentration of gossypetin in plasma and in brain homogenate [56].

5.2.4.Effect of avicularin and gossypetin on Chronic Unpredictable stress (CUS) induced cognition impairment

Bharat Serums and Vaccines Limited, Navi Mumbai, supplied animals. The entire study was completed by using 108 Swiss albino animals. All animals were kept in clean plastic cages. A multi-nutritional pallet diet with purified water *ad libitum* was provided to them, and temperature (22 ± 2^{o}C), relative humidity (75 ± 5%), light/dark cycle (12 hr) were maintained according to CPCSEA guidelines. The Institutional Animal Ethics Committee approved the protocol (approval number- CPCSEA/IAEC/P-40/2019). Gossypetin and avicularin was dissolved in pH 7.4 phosphate buffer solution and sonicated for 30 min whereas DPZ was dissolved in distilled water. Gossypetin were given to the animal through intraperitoneal injection & avicularin was given via oral route. Each group contains 12 animals, and nine groups were formed from all of the animals. Group 1 was given phosphate buffer solution and served as a control group, while Group 2 was subjected to chronic unpredictable stress for 35 days. Groups 3, 4, and 5 were exposed to CUS before being treated with gossypetin (5, 10, & 20 mg/kg) and group 6, 7 and 8 were exposed to CUS before being treated with avicularin (50, 100, & 200 mg/kg), group 9, treated as a standard group, received CUS exposure followed by donepezil hydrochloride treatment [57].

Experimental design

All the animals, excluding the control group, exposed to various stressors. Around ten stressors were selected and assigned randomly for 35 days. The list of stressors and their schedules is mentioned in table no.1. Respective drug treatment was given to the animals for four weeks (from day 22 to day 49). Anxiety and aversive behavior in animals were estimated in the 6th week through various behavioral tests like locomotor test, tail suspension test (TST), forced swim test (FST) and sucrose preference test (SPT).

Learning and memory of animals were analyzed in the 7th week with the help of the Morris water maze (MWM) and passive avoidance test (PAT). On 36th day, the locomotor activity, TST on day 37, FST on day 38 and SPT from day 39 to 42 was performed. Further, PAT was performed on days 43 and 44, MWM test on days 45 to 49. On completion of the behavioural study, all the animals were anesthetized, and blood samples were collected for corticosterone estimation by using ELISA kit. At the end, animals euthanized, and brain samples collected for oxidative stress, neurotransmitters and BDNF levels measurements [58].

5.2.5. Effect of avicularin and gossypetin on Amyloid beta induced Alzheimer's Disease

The National Institute of Biosciences in Pune, India, provided 108 male *Wistar rats* around 3-4 months old weighing between (180-200g). All animals were housed in the institutional animal facility during the acclimation phase and given *ad libitum* food and water (Sohn et al., 2021). According to the CPCSEA guidelines, temperature (22 ± 2ºC), relative humidity (75 ± 5%), light/dark cycle (12 hr), and other conditions were maintained. The Institutional Animal Ethics Committee (IAEC) evaluated and approved the protocol (approval number- CPCSEA/IAEC/P-27/2020).

Experimental design

A total of 108 rats were randomly assigned into 9 groups, and each group contained 12 rats. Group 1 was served as the control group, received 0.5% CMC, group 2 received $A\beta_{1-42}$ peptide via i.c.v. route (disease control group), Group 3, 4, and 5 received avicularin treatment at a dose of (25, 50, and 100 mg/kg, orally). Group 6, 7 and 8 received gossypetin treatment at a dose of (2.5, 5, and 10 mg/kg, *i.p.*) after $A\beta_{1-42}$ peptide (i.c.v.) injection. Group 9 was treated with standard drug donepezil hydrochloride (1

mg/kg, orally) after $A\beta_{1-42}$ peptide (i.c.v.) injection. The activity of avicularin on amyloid-beta mediated Alzheimer's disease was evaluated. On day 0, $A\beta_{1-42}$ peptide was injected unilaterally into the rat hippocampus, and the animals were given drug treatment from the first day to the end of the study. All the behavioral studies were conducted from day 12 to 21, the open field test (OFT) on day 12, the elevated plusmaze test (EPM) on days 13 and 14, the passive avoidance test (PAT) on days 15 and 16, and the Morris water maze test (MWM) from day 17 to 21. After 21 days, all the animals were sacrificed [59].

Stereotaxic surgery

Initially, for anaesthesia, the animals were administered a total of 12 units of ketamine and xylazine solution in a 2:1 ratio through intraperitoneal injection with an insulin syringe. The skull was exposed after the skin was shaved and cleaned using a sterile scalpel. Bregma point was identified, and a hole was drilled using coordinates (AP= 0.8mm, ML=+1.2mm, DV=-3.6mm) and a cannula inserted. $A\beta_{1-42}$ (1µg/µl) was dissolved in phosphate buffer solution (pH7.4) and incubated at 37°C for 3 days to induce aggregation. The cannula was attached to one side of the PE10 tube, and the Hamilton syringe was inserted into the other side of the PE10 tube. A single intracerebroventricular (i.c.v.) injection of the $A\beta_{1-42}$ (4µl) was given to the animals utilising stereotaxic equipment. The wound was stitched up, and to prevent sepsis; all animals were given gentamycin (5 mg/kg, *i.p.*) after surgery [60].

5.2.6. Effect of avicularin and gossypetin on trimethlytin Chloride induced neuroinflammation

The National Institute of Biosciences in Pune, India provided all 90 male Swiss albino mice (20-25g). All of the animals were acclimatized for 7 days, and food and water were

freely available during the entire study. All housing conditions, such as temperature 22 ±1°C and relative humidity of 60% with 12-hour light/dark cycles, were maintained in accordance with CPCSEA guidelines. The procedure was assessed and approved by the Institutional Animal Ethics Committee (IAEC) (approval number- CPCSEA/IAEC/P-27/2020).

Experimental design

After acclimatization, the animals were divided into 9 groups, 10 animals in each group. Group 1 served as the control group, Group 2 as the TMT exposed mice (disease control group), and Groups 3, 4, and 5 as the avicularin (50, 100, and 200 mg/kg) groups. Groups 6, 7, and 8 as the gossypetin (5, 10, and 20 mg/kg) groups. Group 9 was given donepezil hydrochloride. TMT was administered to the animals on day 21. From day 22 to day 28, a passive avoidance test and a Morris water maze test were used to measure cognition activity in rats. At the end of the study, all the animals were euthanized using the Co_2 asphyxiation procedure. Hippocampus tissue was removed and kept at -80ºC. Further biochemical analysis and GSK-3β levels measured [61].

6. RESULTS

Initially, we have investigated the toxicological profile of avicularin and gossypetin via the intragastric route. The study focused on measuring the safety and effect of the acute and repeated dose of avicularin and gossypetin in animals. Earlier results specified the anti-depressant, anti-inflammatory, and antioxidant activity of avicularin and anxiolytic, antidepressant and anti-oxidant properties of gossypetin. Some researchers also demonstrated beneficial role of avicularin in multiple studies. Furthermore, computation studies revealed that, safety of avicularin and gossypetin, hence acute toxicity study of avicularin was conducted at 300, 2000, 5000 mg/kg and gossypetin at 300 &

2000mg/kg. Despite the good therapeutic efficacy of avicularin and gossypetin, there is no systematic toxicity study has been conducted to date. Hence, a toxicity study is urgently needed so that the appropriate dose can be used in animal studies to demonstrate its potential effect. Additionally, by considering its potential actions of avicularin and gossypetin and to investigate the possible action of avicularin and gossypetin we have conducted toxicity study of avicularin. In the acute and repeated toxicity study, avicularin and gossypetin treated rats did not display any change in body weight, food and water intake, behavioral parameters, external physical appearance, and mortality.

This condition indicates that avicularin did not produce any mortality, toxic signs.

Further, we have also performed pharmacokinetic study of avicularin and gossypetin.

All the processed samples analysed in LC-MS/MS and all the data evaluated by using PK solver software. The half-life of avicularin was found to be 287 ± 12.57 min, time required to reach maximum concentration was found to be 240 ± 11.22 min, concentration maximum was found to be 661.47 ± 54 ng/ml and area under the curve was 242906.65 ± 1127.27ng/ml/min.

The concentration of avicularin in brain was also evaluated. The half-life of avicularin was found to be 160 ± 14.96 min, time required to reach maximum concentration was found to be 360 ± 21.36 min, concentration maximum was found to be 412.08 ± 88.96 ng/ml and area under the curve was 220278.87 ± 1096.32 ng/ml/min.

The half-life of gossypetin was found to be 448 ± 13.28 min, time required to reach maximum concentration was found to be 360 ± 19.62 min, concentration maximum was found to be 669 ± 59 ng/ml and area under the curve was 397379.225 ± 1031.04ng/ml/min.

The concentration of gossypetin in brain was also evaluated. The half-life of gossypetin was found to be 415 ± 22.32 min, time required to reach maximum concentration was found to be 540 ± 29.16 min, concentration maximum was found to be 555.06 ± 81.26 ng/ml and area under the curve was 413724.6 ± 1145.84 ng/ml/min.

Further we have evaluated the activity of avicularin and gossypetin against CUS induced cognition impairment, Chronic unpredictable stress mainly causes cognition impairment by affecting HPA axis function. This HPA axis dysfunction also correlated with KYN pathway. This research study mainly evaluated the neuroprotective effect of gossypetin against cognitive dysfunction via inhibiting undesirable effects of KYN pathway. Primarily, to assess gossypetin activity against impaired cognition, a preliminary efficacy study was conducted, and the neuroprotective activity of gossypetin was assessed at 5, 10, and 20 mg/kg based on the pilot studies results.

Various behavioral tests were conducted in the sixth and seventh weeks to examine the impact of chronic stressors on animal behavior. An open field test was used to assess the effects of CUS and gossypetin and avicularin on locomotor function. The open-field test is the most widely used method for evaluating mouse mobility and exploratory behavior. When compared to other groups, CUS-exposed mice showed considerably reduced locomotor activity and rearing frequency. TST and FST determined the effect of gossypetin on stress circumstances and behavioral dejection in animals. CUS has been shown to enhance immobility behavior in prior research. Similarly, in our study, CUS exposed mice showed significantly increased immobility activity, whereas significant reduction in immobility time was observed gossypetin and avicularin-treated mice when compared with CUS-exposed mice.

Furthermore, stressed animals showed a lack of preference for a palatable solution (sucrose solution) in the sucrose preference test which was used to quantify anhedonia

activity of mice. The current research discovered that stressed animals showed a significant decrease in preference for a palatable solution. At the same time, gossypetin treated 10 and 20 mg/kg and avicularin at 100 & 200 mg/kg mice displayed a substantial increase in sucrose preference ratio compared to mice exposed to CUS.

Depressive and anxiety like symptoms were observed in TST, FST, SPT behavioral tests in stressed animals due to excessive cortisol induced production of IDO. This IDO enzyme interferes with tryptophan metabolism by limiting BH4 activity in the axonic end bulb of the neuron and decreases the availability of monamines in the synaptic cleft, resulting in repolarization of the cell membrane and produces depressive-like symptoms in animals Whereas, gossypetin and avicularin treatment successfully dimishes depressive like symptoms in animals by elevating monoamines levels in the synaptic cleft and blocking release of excessive cortisol from adrenal gland.

Furthermore, MWM and PAT are frequently utilized paradigm in animals to assess cognitive activity. In MWM and PAT, stressed animals were unable to recognize target quadrant and shock area zone due to cognitive dysfunction and also showed decreased transfer and escape latency in comparison with control group. According to previous studies, activated KYN pathway cause NMDAR (N-methyl D-aspartate receptor) activation through increasing levels of quinolinic acid in microglia. This NMDAR activation increase excitotoxicity in neuronal cells and causing release of excessive Ca^{2+} ions and leads to neuronal cell death. This has negative impact on sensory and motor function as well cognitive functions of animals. As a result, CUS exposed mice showed alteration in memory, learning and recognition ability. In contrast, gossypetin and avicularin treated mice showed increased spatial learning activity.

The HPA axis is disrupted by chronic unpredictable stress, which leads the anterior pituitary gland to generate more adrenocorticotropic hormone (ACTH), causing the

adrenal cortex to synthesis too much corticosterone. Moreover, corticosterone is a crucial indicator of stress; the amount of corticosterone in serum may be used to assess the stressed condition. The control group compared to CUS-exposed mice had a substantial rise in serum corticosterone levels. In contrast, the gossypetin and avicularin treated group had lower corticosterone levels. This suggests that gossypetin and avicularin helps to lower corticosterone levels in the blood.

Further, chronic stress showed enhancement in the production of reactive oxygen species (ROS). These highly reactive metabolites cause lipid peroxidation by reacting with unsaturated fatty acids in the phospholipid membrane. This interrupts the electron transport route, causing cellular damage and ultimately reducing neuronal life span. Anti-oxidant enzymes such as SOD, GSH, and catalase play an essential part in the antioxidant defence system. Environmental or chronic stress disturbs the anti-oxidant mechanism. Hence, CUS exposed mice showed lower levels of antioxidant enzymes.

Apart from this, excessive malondialdehyde is a result of the lipid peroxidation process. Our findings support an earlier study, demonstrated a reduction in SOD, GSH, catalase, and an increased in MDA level in CUS-exposed mice under stressful conditions. According to a published study, gossypetin and avicularin has free radical scavenging activity and acts as an anti-oxidant agent. We also found an elevated SOD, GSH, and catalase level and decreased MDA level upon gossypetin and avicularin treatment.

Furthermore, high corticosterone levels limit BH4 production, which is a necessary enzyme for neurotransmitter synthesis. This event decreases neurotransmitter availability in the synaptic cleft, reducing monoamines levels and binding with receptors. As a result, the levels of serotonin and norepinephrine were also examined. In our findings, CUS-exposed animals had lower levels of serotonin and norepinephrine, which were restored by gossypetin and avicularin therapy. Furthermore, CUS-exposed

animals had altered tryptophan metabolism and elevated kynurenine levels, resulting in an increased Kyn/Try ratio and a reduced 5-HT/Try ratio. Gossypetin and avicularin treated mice successfully reduced high cortisol and monoamime levels, balancing the ratio of Kyn/Try and 5-HT/Try. Brain derived neurotrophic factor promotes neurogenesis. According to earlier studies, CUS induced excessive quinolinic acid decreases BDNF expression in the hippocampus and the prefrontal cortex. Chronic stress also disturbs BDNF/TrkB binding and inhibit the neurogenesis process. All these events are reversed by gossypetin and avicularin therapy, which increases BDNF expression in the hippocampus and prefrontal cortex by halting the excessive QA production.

Further study emphasises on the neuroprotective effects of avicularin and gossypetin against amyloid beta-induced Alzheimer's disease. Various factors are involved in Alzheimer's disease progression, but amyloid-beta aggregates are the main culprit for neuronal damage resulting in neuronal death and cognition impairment.

Initially, several behavioral studies were conducted to assess the cognition enhancement activity of avicularin. Locomotory and exploratory behavior of rats were measured by using OFT. It was perceived that avicularin treated animals showed exploratory behavior and did not show any signs of depression (lack of movement) compared with the disease control group. Similarly, EPM and PAT were conducted to detect retention memory and the ability of animals to remember the aversive stimulus. The test confirmed that avicularin and gossypetin treated rats have a better aptitude for recognition and cognition when compared with the disease control group.

MWM test is an established model for spatial learning and memory measurement. Our study observed that disease control animals took a long time to reach the platform compared with control treated rats. This significant increase in escape latency indicates

impaired cognition in the disease control group. Avicularin and gossypetin treated animals could reverse the amyloid-beta effect and showed decreased escape latency time.

The correlation between amyloid-beta and oxidative stress has been reported by many researchers. Amyloid-beta causes the production of excessive reactive oxygen species (ROS). This excessive produced ROS interacts with nitric oxide (NO) and causes excessive release of reactive nitrogen species, leading to protein carbonylation, nucleic acid oxidation, and glycoxylation. All these events contribute to AD progression. In our study, avicularin and gossypetin treated animals showed elevated levels of catalase, SOD, GSH activity, and diminished MDA activity; these results are consistent with previous antioxidant results of avicularin and gossypetin and confirm that avicularin and gossypetin can reduce amyloid beta-induced oxidative stress.

Elevated oxidative stress also alters the level of acetylcholinesterase enzyme activity. The acetylcholinesterase enzyme is primarily responsible for reducing acetylcholine supply in the synaptic cleft and preventing further synaptic transmission. Acetylcholinesterase enzyme activity was evaluated to measure the level of acetylcholine in the hippocampus and cortex area of the brain. The hydroxylation activity of acetylcholine iodide was measured with the help of UV spectroscopy. It was observed that the $A\beta_{1-42}$ exposed group showed an elevated level of acetylcholinesterase activity, and avicularin and gossypetin treated rats displayed a significant decrease in acetylcholinesterase activity. This indicates that, since avicularin and gossypetin reduces the acetylcholinesterase enzyme activity, the availability of acetylcholine neurotransmitters in the synaptic cleft would rise resulting in cognitive-enhancing action.

Furthermore, amyloid-beta aggregates are responsible for RAGE activation, which causes activation of the NF-κB signaling pathway and the release of inflammatory mediators. The anti-inflammatory action of avicularin and gossypetin has previously been documented; nevertheless, we demonstrated possible avicularin and gossypetin activity against amyloid beta-induced neuroinflammation in this experimental study. We observed that our data is consistent with previously reported data.

Amyloid beta is the critical predisposing factor for AD progression, and BDNF is crucial for neurogenesis. Immunohistochemistry was performed to see how avicularin and gossypetin affects brain-derived neurotrophic factors (BDNF) and amyloid-beta levels in the brain. In line with previous research, $A\beta_{1-42}$ exposed rats had lower BDNF expression in the hippocampus and cortex tissue, negatively impacting the neurogenesis process. Furthermore, BDNF is also responsible for RAGE deactivation, which suppresses the RAGE-mediated oxidative stress and inflammation. It was observed that avicularin and gossypetin is beneficial in increasing BDNF levels, ultimately reducing excessive inflammatory response, oxidative stress, and support neuroprotection. Furthermore, to elaborate on the neuroprotective effect of avicularin and gossypetin, we have also measured the expression of amyloid-beta in the hippocampus and cortex region of the brain. Interestingly, avicularin and gossypetin was able to reduce the expression of amyloid-beta in both the region of the brain and showed neuroprotective effect against amyloid-beta induce impaired cognition.

Further the activity of avicularin and gossypetin has been evaluated against TMT induced neuroinflammation and GSK-3β levels in the brain.

Neurofibrillary tangles are a prominent histopathological feature that are made up of aberrant fibrils observed in neurons. The primary goal of this study is to assess the activity of avicularin and gossypetin against GSK-3β-induced neurofibrillary tangle

development. Several literature searches revealed that trimethyltin chloride is a highly neurotoxic compound that raises GSK-3β levels in the brain, causing brain malfunctioning. Considering TMT's effect on the brain, we used TMT as an inducing agent for our study.

The dosage of TMT was optimised at 2.6 mg/kg during our standardisation study and literature search. Further, avicularin and gossypetin treatment was given to the all mice for 28 days. In between 22 to 28 day we have performed two cognition tests (MWM & PAT), both the tests suggest that avicularin and gossypetin treated animals remember shock area zone and can avoid shock region by spending more time light compartment in PAT and also finds platform early in MWM test, when compared with disease control group. Statistical analysis confirms that treatment group showed enhanced transfer and decrease escape latency.

TMT also promotes excessive oxidative stress and microglia activation. As a result, we examined the levels of SOD, GSH, Catalase, and MDA in the hippocampus region of the brain. In our research, we discovered that avicularin and gossypetin possesses radical scavenging action and boosts anti-oxidant species levels in the brain while decreasing free radical activity. ELISA kits were used to quantify the levels of pro-inflammatory cytokines from activated microglia. When compared to the disease control group, the levels of IL-6, IL-1β, and TNF-α were shown to be lower in the avicularin and gossypetin treated animals. Based on these findings, we can state that avicularin and gossypetin is a potential anti-oxidant and anti-inflammatory phytoconstituent. Furthermore, our findings are consistent with earlier research study. At the end of study, to conclude action of avicularin and gossypetin against GSK-3β, we have performed immunohistochemistry. Results state that, avicularin and gossypetin has ability to reverse high levels of GSK-3β produced by TMT. Avicularin and gossypetin treated

mice (50 & 100 mg/kg) showed lower expression of GSK-3β levels in the hippocampus, which ultimately state that avicularin and gossypetin mitigates TMT induced hippocampal neurodegeneration.

CONCLUSIONS

Initially, from the results, we can conclude that, avicularin and gossypetin are safe phytoconstituents and did not produce any mortality and toxic signs in animals. After that gossypetin and avicularin evaluated against CUS induced cognition impairment. Based on the findings, it is concluded that gossypetin and avicularin can effectively cure chronic unpredictable stress-induced cognition impairment. Gossypetin and avicularin therapy also improved the behavioural parameters, decreased oxidative stress and corticosterone levels and increased neurotransmitter levels. Gossypetin-treated mice at 10 and 20 mg/kg and avicularin at 100 and 200 mg/kg displayed an increase in BDNF expression in the brain. Moreover, gossypetin and avicularin possess potential to act as a neuroprotective phytochemical agent against cognition impairment caused by CUS.

Further from amyloid beta model, we may conclude that avicularin and gossypetin can improve learning and cognitive behavior and reverse the negative effects of amyloid beta, including inflammatory response and oxidative stress. Furthermore, the findings reveal that avicularin can halt AD progression by targeting BDNF and amyloid-beta levels in the brain, suggesting that avicularin and gossypetin could be used for Alzheimer's disease treatment.

The hippocampus is important in long-term episodic memory development, organisation, and recognition. TMT is a hazardous organotin chemical that disrupts hippocampus function by increasing GSK-3β levels. Preliminary pharmacokinetic studies show that avicularin and gossypetin has the capacity to pass the blood-brain

barrier (BBB). Furthermore, behavioural investigations validate avicularin and gossypetin cognition-enhancing action. Furthermore, avicularin and gossypetin therapy reduced TMT-induced oxidative stress and inflammatory mediators. To confirm neuroprotective activity of avicularin and gossypetin against TMT-induced GSK-3β, we relied on immunohistochemistry data, which support our hypothesis that avicularin and gossypetin has neuroprotective action against TMT-induced hippocampus neurodegeneration by reducing GSK-3β levels.

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